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Report on the Bacteria Source Tracking Project

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This research is a product of the Department of Biology faculty at Indiana University-Purdue University Fort Wayne.

Report on the

Bacteria Source Tracking Project

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Summary

The Bacteria Source Tracking (BST) project was begun in 2001 and concluded in 2004. Antibiotic Resistance Analysis (ARA) was used in an attempt to determine the source of bacterial contamination in the St. Joseph River Watershed. The St. Joseph River is the largest tributary to the Maumee River system, which empties into Lake Erie at Toledo, Ohio. The St. Joseph River and several of its tributaries, including the largest, Cedar Creek, are on Indiana's 303(d) list of impaired waters for *E. coli*.

This research endeavor included development and refinement of a database particular to Northeast Indiana of known source patterns of resistance to antibiotics for humans, horses, beef and dairy cattle, deer, geese, hogs and domestic pets. Enterococci were extracted from water samples and tested against this database to determine sources of the contaminant.

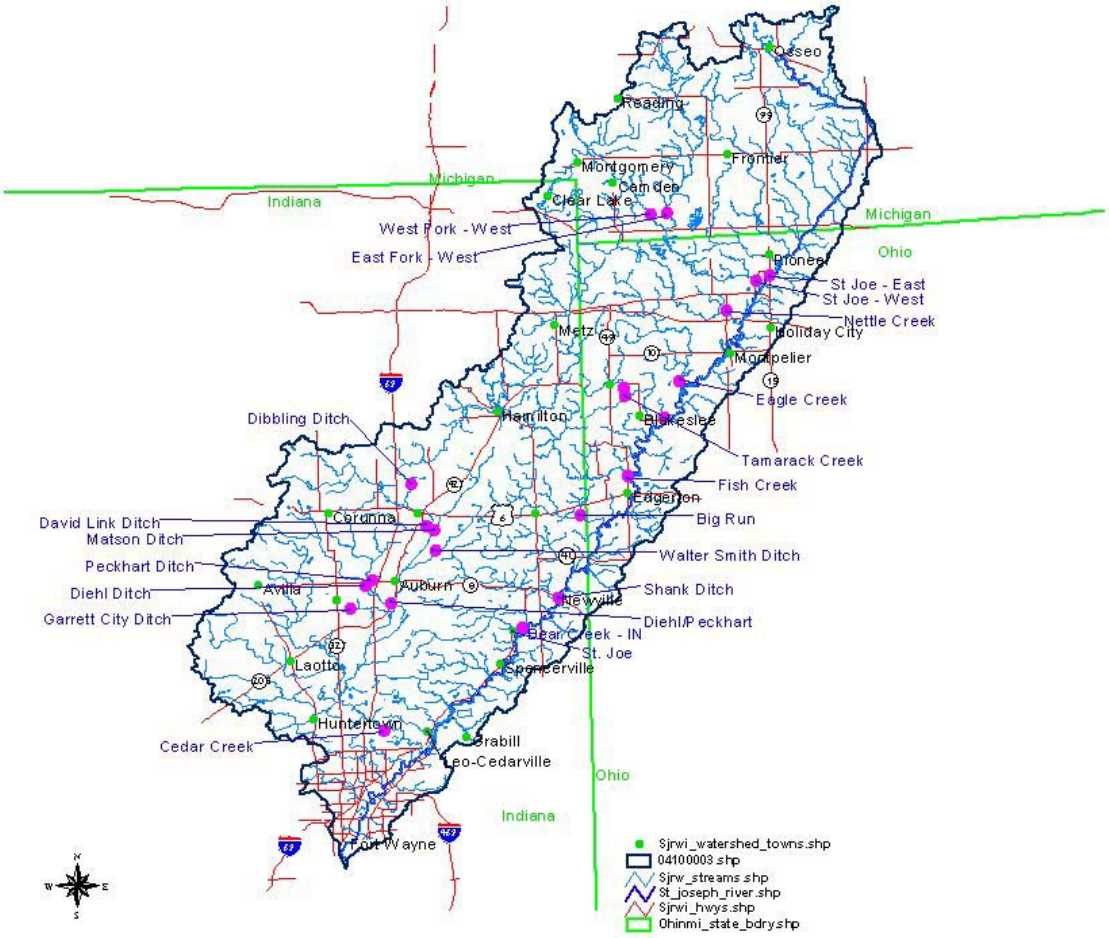
Results of the research indicates that wildlife, particularly geese, make a significant (greater than 50%) contribution to the bacterial pollution in this watershed. The human contribution of fecal contamination is localized to particular sub-watersheds and is generally low. Livestock (beef, dairy and swine) contribute little to the overall fecal pollution of the St. Joseph River watershed.

Significant contribution is shown from horses; however there is some question whether there is interference with horse from another source of contribution. It is known that this possible interference does not come from human sources.

The knowledge of land uses is an essential component of bacteria source tracking through the use of ARA.

St. Joseph River Watershed Initiative

2003 Water Sampling Stations



Introduction

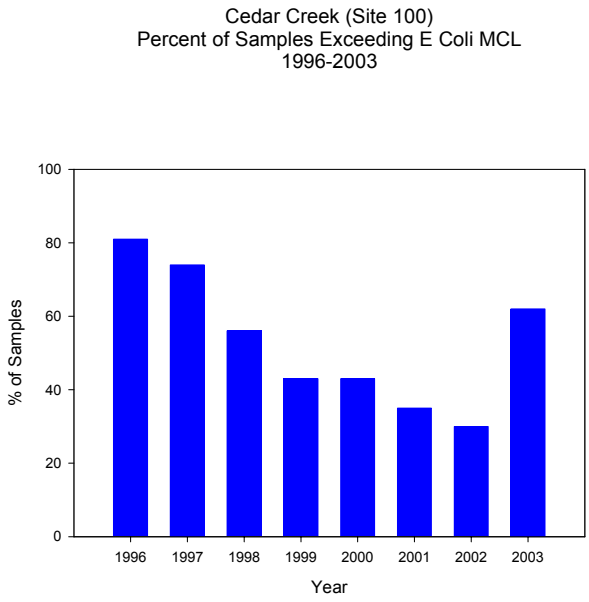
Over the past decade, there has been an increasing concern over the presence of *E. coli* in lakes and streams in Indiana and nationwide. *E. coli* is a bacterium that is found in human and animal fecal material in relative high numbers. Although most strains of *E. coli* do not cause disease in humans, the bacterium is a reliable indicator of contamination by human and/or animal feces which could be a threat to human health.

E. coli can enter receiving waters from three known routes: overflow from combined sewer systems, drainage from septic tanks and contributions from animal fecal material. The first two situations represent direct health concerns due to potential exposure to human disease-causing organisms by what is known as the oral-fecal route. The latter situation is also significant due to the potential for transfer of pathogens from animals to humans.

Elevated levels of *E. coli* in the waters of the St. Joseph River and its tributaries have long been identified through monitoring conducted by the St. Joseph River Watershed Initiative (SJRWI), the Fort Wayne - Allen County Department of Health, and the Indiana Department of Environmental Management (IDEM). When proposing remediation activities for watershed streams, it is important to be able to distinguish between animal contributions and human contributions to the total *E. coli* load to receiving waters.

However, to accurately determine whether the sources of contamination are human (from failing septic systems or sewage treatment facilities), domestic animal, livestock, or wildlife, additional research was deemed necessary. The Bacteria Source Tracking (BST) project begun in 2001 was an effort to produce a database of characteristics of known fecal contaminants which could then be compared to water samples taken from throughout the watershed.

The SJRWI has collected grab samples of the waters of the St. Joseph River and several tributaries since 1996. The graph below illustrates the percentage of SJRWI weekly grab samples that exceeded the recreational standard of 235 colonies per 100 ml water for E. coli from 1996-2003 at Site 100, the confluence of Cedar Creek with the St. Joseph River, just north of Fort Wayne.



While E. coli is the most common indicator of fecal contamination, other bacteria, such as fecal enterococci are also used as indicators. Currently available methods for pinpointing the source of fecal contamination in the environment fall into two major categories: the use of antibiotic resistance analysis (ARA) and the use of molecular biological techniques. The latter have proved their worth in identification of pathogenic strains in cases of food borne disease where the number of samples needed is limited. However, they are time consuming and expensive. Furthermore, a database linking individual strains of fecal bacteria and sources of pollution (i.e. human, waterfowl, swine, cow, etc.) does not exist. Development of such a database would be prohibitively costly and time consuming. The use of ARA, however, offers speed, reproducibility, low expense and a proven track record.

The BST project was initiated in spring of 2001 with a grant to the SJRWI from the Fort Wayne Community Foundation, which funded setup of the database and baseline sampling. The project was continued when the SJRWI was awarded a Section 319 grant in 2002 which funded the project in a joint effort with the Biological Sciences Department of Indiana University – Purdue University Fort Wayne (IPFW). Dr. Deborah Ross is the chief investigator for the project.

Year 2001: Setting up the BST Project

In the antibiotic resistance analysis method, which utilizes replica plating technology, bacterial strains are isolated from the environment, and characterized as to their sensitivity to a range of antibiotics. The basis for this method assumes that if bacteria have been exposed to a given antibiotic, they will develop resistance to it; if they have not been exposed, they will not be resistant. Thus the growth pattern of

bacterial strains from water is matched against standard strains from known sources. Such a database was developed by researchers in Virginia, using fecal enterococci as the indicator of choice. With this database, researchers were able to identify inputs of fecal bacteria from a farm as resulting from uncontrolled access of cattle to the stream in question, and the remediation of this situation, installation of fencing, was simple and expedient. Researchers in Florida have used this method in subtropical waters.

The objectives of the SJRWI study were to develop a database similar to those used in Virginia and Florida for the St. Joseph (Maumee) River watershed. It was initially proposed to make a determination whether or not the Virginia database was suitable for the situation in northern Indiana, as planners felt that the development of a local database might be too time consuming to complete within the proposed time limits. Upon consultation with the Virginia researchers, planners decided to go ahead and develop the database from sources specific to northern Indiana. This guarantees the applicability of our database to our own watershed. The concerns regarding the time frame were due to an overestimation of the time required to determine the antibiotic resistance patterns of strains of enterococci from known sources.

The first step in development of the database was to determine the significant sources of fecal contamination in the St. Joseph River watershed. Advice from the SJRWI management and county extension agents resulted in the following list of sources: human, domestic pet, swine, beef cattle, dairy cattle, horses and wildlife (raccoons, deer, etc). A sampling schedule was developed which would result in the collection of samples and their return to the laboratory where they could be immediately sampled for fecal enterococci.

The initial sampling consisted of dilution of a weighed amount of fecal material (10 g) in physiological saline and plating of a volume (0.1 ml) of the appropriate dilutions onto a bacteriological medium designed to enhance to growth of enterococci. Dilution of the sample is necessary into order to obtain separation of bacterial cells within the fecal material such that individual cells will grow into isolated, defined colonies which appear red. These colonies can then be transferred into individual wells on a microwell plate. Each well contains a volume of 0.2 ml of medium which will turn black when enterococci are grown. Thus, individual bacterial cells can be cultured and tracked. Following growth in the microwells, a replica plating device can be used to transfer the bacteria from the microwells to bacteriological growth medium containing various concentrations of antibiotics.

To build the initial SJRWI database, from one to five concentrations of nine antibiotics were used for a total of 30 combinations. Because of the small volumes involved and the use of the replica plater, 96 bacterial strains can be inoculated onto the 30 media in a matter of 15 to 20 minutes. After two days' growth, strains are scored for growth or nongrowth on each of the antibiotic concentrations. This method worked so rapidly that it was possible to test over 1,000 bacterial strains within six weeks. The second step in the process is to enter each strain into a computer program which is capable of performing discriminant analysis. JMP IN was selected because the researchers in Virginia had used this program and the use of it on the local project would make our two databases compatible. In this statistical program, analysis of variance is first performed on the dataset, then strains from known sources are compared and grouped based on similarity. The goal is to be able to separate strains from a given source based on the susceptibility to each of the nine antibiotics.

Year 2002: Refining the Database

The goals for the project in 2002 were twofold:

- Expand the existing database to include approximately 1,000 strains from identified sources;
- Survey the SJRWI monitoring sites and identify the sources of fecal contamination at those sites.

To address the first goal, the investigator visited county 4H fairs in Dekalb County, Indiana, Williams County, Ohio and Hillsdale County, Michigan and took samples of fecal material from horses, swine, dairy and beef cattle. Several samples from each source were pooled to form one composite sample. Samples were stored under refrigeration during transport. Upon return to the lab, enterococci were isolated and characterized according to the established procedure (Appendix G). In addition, the investigator visited animal shelters in Fort Wayne (Fort Wayne Animal Control and Allen County S.P.C.A.). Cat and dog samples were taken at each location. Multiple samples of each animal type were pooled into one composite sample of each type. Samples were stored under refrigeration during transport and analyzed as indicated on the attached protocol. Human samples were collected by the Indiana Department of Transportation (INDOT) at roadside rest areas along Interstate 69. Four samples of influent sewage were collected and brought to the laboratory at Indiana-Purdue University Fort Wayne by INDOT personnel. They were analyzed as described. Addition of microorganisms from these sources composed a database of over 1,000 strains which was used in the analysis of the season's water samples.

To address the second goal, each of the 18 weekly monitoring sites established by the St. Joseph River Watershed Initiative within the watershed was visited twice, once during late July/early August and again during late August/early September. An additional site on the St. Joseph River near the City of Fort Wayne's water intake at the St. Joseph dam was also sampled. Technicians performing BST followed the SJRWI's technician on the sampling route and collected water samples for BST at approximately the same time on the same day as the SJRWI's water samples were collected. The BST technicians did not take measurements of stream depth, and therefore water level information has been supplied by the SJRWI's data (measurement of bridge to water surface). Five to six water samples were taken on each sampling day and stored under refrigeration until their return to the lab where they were processed according to the established protocol.

Data from the 2002 monitoring sites are shown in a spreadsheet in Appendix A to this document. Data are reported in terms of percentages of isolated strains matching one of five source categories: livestock (beef and dairy cattle, and swine), horse, human, domestic pet and geese. Several conclusions emerge from the 2002 sampling data.

- The livestock contribution is low throughout the watershed.

- The human contribution is generally negligible or low throughout the watershed.

- Geese make up a significant source of pollution, particularly in the set of samples collected in late summer.

- The two sets of samples from a given sampling site do not necessarily agree, suggesting that sources can change with time. This suggests that a single sample may not be representative for

regulatory purposes.

The testing carried out during the summer and fall of 2002 resulted in further refinement of the ability to positively identify fecal contaminant sources. The following table shows the rate of correct classification of each fecal source.

Fecal Contaminant Source	Rate of Correct Classification
Geese	89%
Domestic pets	86%
Swine	83%
Beef cattle	71%
Dairy cattle	58%
Horse	63%
Human	58%

After the analysis for this season was completed, a significant overlap in identifying horse, dairy cattle and human sources remained to be further refined. Additionally, there was interest in two additional factors which have been reported to affect the analysis of bacteria in the stream: flow rate and distance downstream from a contaminant source.

Year 2003: Time and Dilution Studies

The goals for the project in year 2003 were:

- To further refine the database
- To sample each of the SJRWI monitoring sites
- To perform more extensive sampling in certain key sub-watersheds in order to determine whether sampling closer to sources would change the source distribution
- To perform multiple samples on a subset (five) of the SJRWI's monitoring sites to determine the effect of time during the sampling season as a factor in the source distribution

To further refine the database, additions were made by gathering fecal samples of livestock and horses at the county fairs in Allen and Dekalb counties in Indiana and Williams county in Ohio, as well as by collecting samples of the fecal material of domestic pets from the Fort Wayne Animal Control and the Dekalb County Animal Shelter. Additional human waste samples were procured by sampling additional wastewater supplied by the INDOT from roadside rest areas.

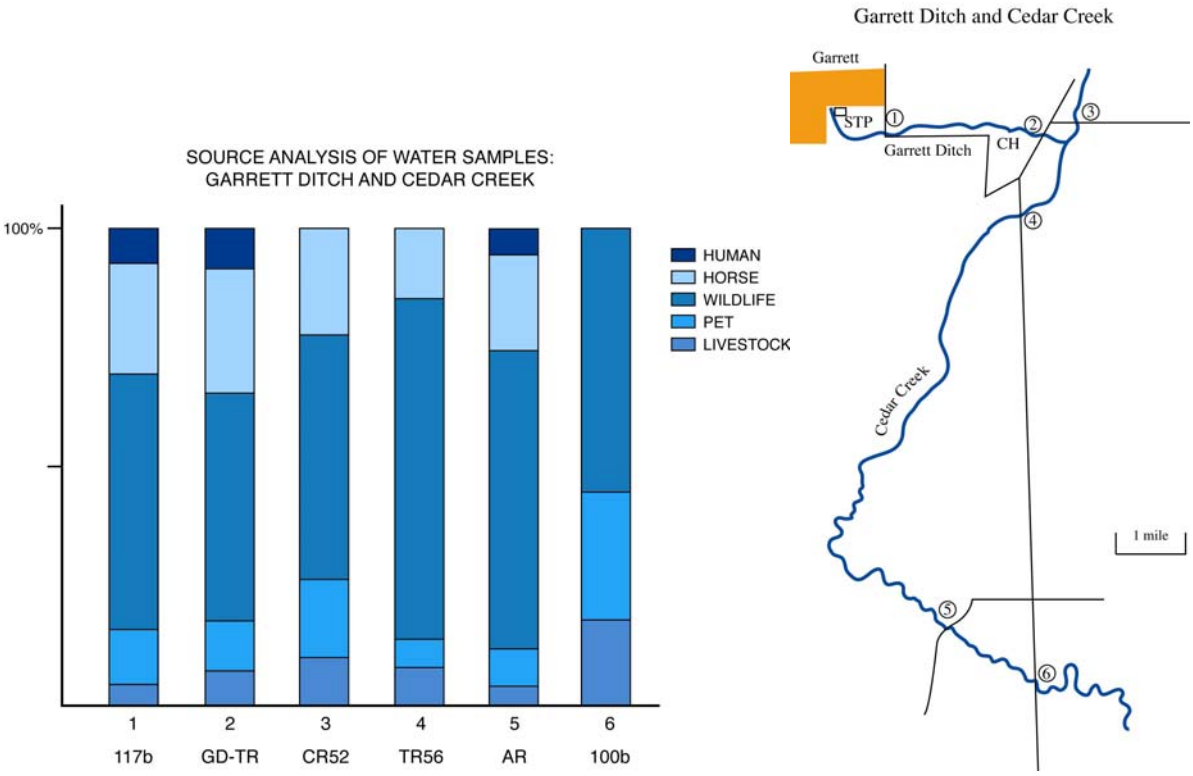
To meet our goal of continuation of overview sampling of the watershed, each of the SJRWI's 2003 monitoring sites were sampled once. During this season, BST technicians made an independent sampling trip on the same day as the SJRWI's water sampling technician. A bridge-to-water measurement was taken by the BST technician so that changes in water level between the two sampling times could be determined. Samples were maintained on ice until their return to the lab and processed as described above. Data from source analysis of these samples is compiled in the spreadsheet in Appendix B of this document.

To meet our goal of determining how distance from the source would affect the distribution of bacterial

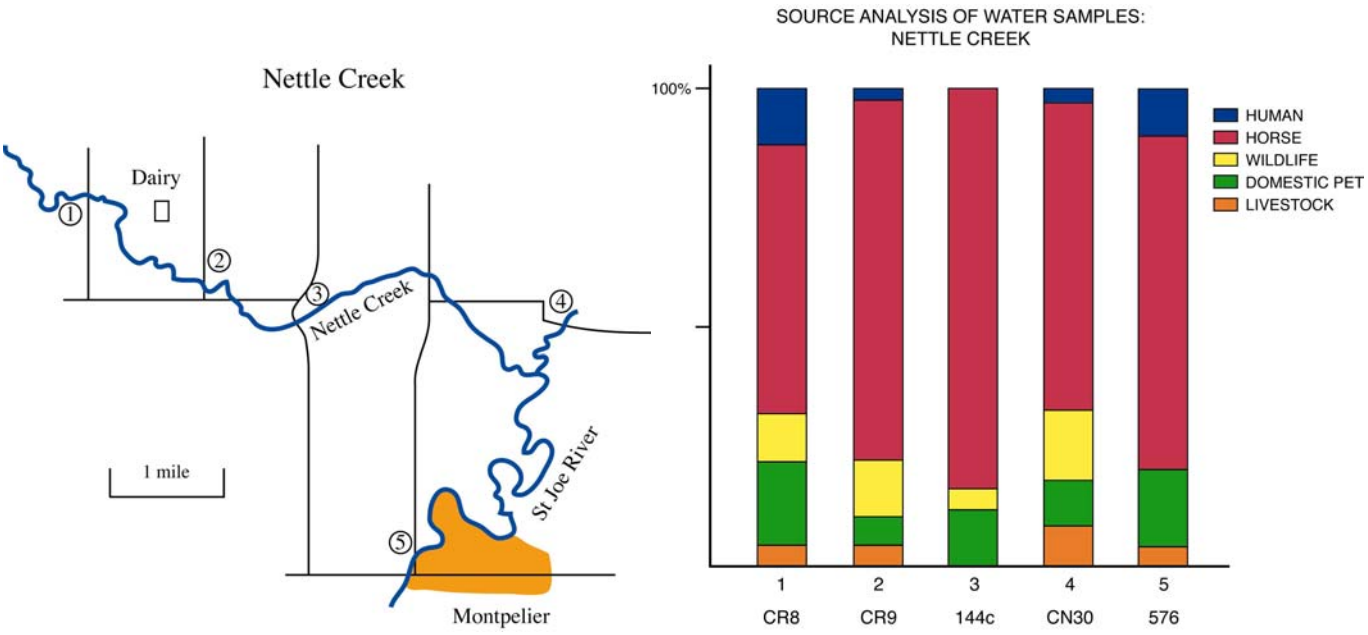
contamination, four sub-watersheds were selected for more detailed sampling. Sites near urban areas where sewer overflow could impact the water quality were identified, as well as several locations where land use information indicated that particular sources of fecal bacteria were likely to impact the water (i.e. a livestock operation located near a stream or ditch). The sampling regime was designed to take samples immediately upstream of each site, and then at various distances downstream of the site to determine how far each source might be expected to impact the results of antibiotic resistance analysis. The following sites were chosen for this analysis: Dibbling Ditch and Garrett Ditch in DeKalb county, Indiana, both in the Cedar Creek sub-watershed; Nettle Creek in Williams county, Ohio, part of the Nettle Creek sub-watershed; and tributaries to the St. Joseph River in the vicinity of Grabill, Indiana in Allen county, located in the Lower St. Joseph and Bear Creek sub-watesheds.

Dibbling Ditch was chosen because of small livestock (cattle) operations located in the immediate area. Samples were taken above the cattle farm, immediately below the cattle farm, at the SJRWI's monitoring site on the Dibbling Ditch (Site 143) and at two sites on the Cedar Creek below the confluence of the Dibbling Ditch with the Cedar. At none of the sites was beef or dairy cattle significant as a source of *E. coli* contribution. The most significant source identified at these locations was horse. Our knowledge of land use in this sub-watershed indicates that there are not significant numbers of horses present, and this analysis, therefore, raises the question of interference between horse signature and that of another source(s).

Garrett City Ditch was selected for detailed sampling because in 2003 and for several years previously, the city's sewage treatment plant had come under significant scrutiny by IDEM due to violations to their NPDES permit and was undergoing expansion and upgrade. The SJRWI's water quality monitoring project had recorded very high levels of *E. coli* in the Garrett City Ditch downstream of the plant during 2003. BST technicians took two samples along the Garrett City Ditch, (one of which was at the SJRWI's monitoring site 117), one sample from the Cedar Creek above the confluence of City Ditch with the Cedar Creek, and a sample each from two locations downstream of that confluence (one of which was the SJRWI's Site 100 just above the Cedar's confluence with the St. Joseph River).

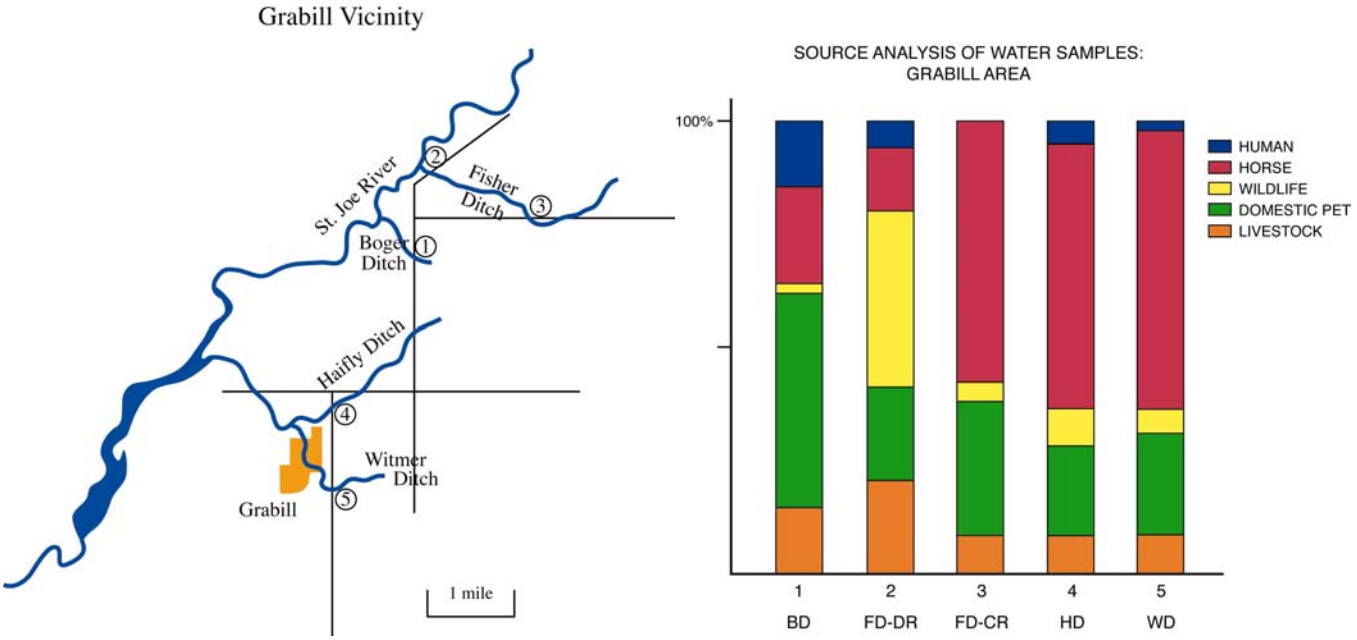


All of these samples indicated that wildfowl were the most significant source of fecal contamination to this sub-watershed. In spite of the problems Garrett was experiencing with capacity at its municipal treatment plant, human enterococci were only found at a level of approximately 10% in the Garrett City Ditch, suggesting that problems from the sewage treatment plant may have been intermittent.



The third location, Nettle Creek, was selected because of a large dairy operation in this sub-watershed. A

sampling site was selected on Nettle Creek above the dairy operation and two on the creek below the dairy, one of which was the SJRWI's monitoring site144. Two locations on the St. Joseph River were also selected, one above and one below the river's confluence with Nettle Creek. Livestock as a source of bacterial contamination varied between about 5 and 15% at these sampling locations, and humans as a source varied between 0 to 15%. The most significant source to the fecal load was horse, suggesting again that there might be interference between the horse signature and another source.



At the fourth location, tributaries to the St. Joseph River in the Grabill, Indiana vicinity were selected to measure pollutant loading in the lower St. Joseph sub-watershed. This area has a significant Amish population and has a larger number of horses than non-Amish areas of the watershed. The SJRWI does not regularly sample the river in this sub-watershed and has not collected data regarding the levels of *E. coli* generally present in these streams. Two sampling sites were selected on Fisher Ditch, one on Boger Ditch, one on Haifly Ditch and one on Witmer Ditch. Domestic pets and horses were found to be the most significant sources of fecal contamination in this area. Livestock and human sources varied, but were not consistently found as major sources.

To meet our final goal, determining the effect of time over the sampling season as a factor in the source distribution, five of the SJRWI's sampling sites were selected for repeated sampling: Big Run, (Site 127), Garrett Ditch (Site 117), Dibbling Ditch (Site 143), Nettle Creek (Site 144) and Diehl Ditch (Site 126). Since these sites were sampled over three years, they will be discussed in the final section of this report.

Conclusions from the year 2003 analysis are similar to those of the 2002 sampling season, and indicate that livestock and human contributions to fecal contamination are low (generally under 10%) while the contribution from wildfowl is considerable. Horses as a source of pollution also appear to be significant.

Year 2004: Trends

This was the final season of sampling for this project, and all sampling work was scheduled to be completed by June 30 of the year. The goals of the 2004 sampling season included:

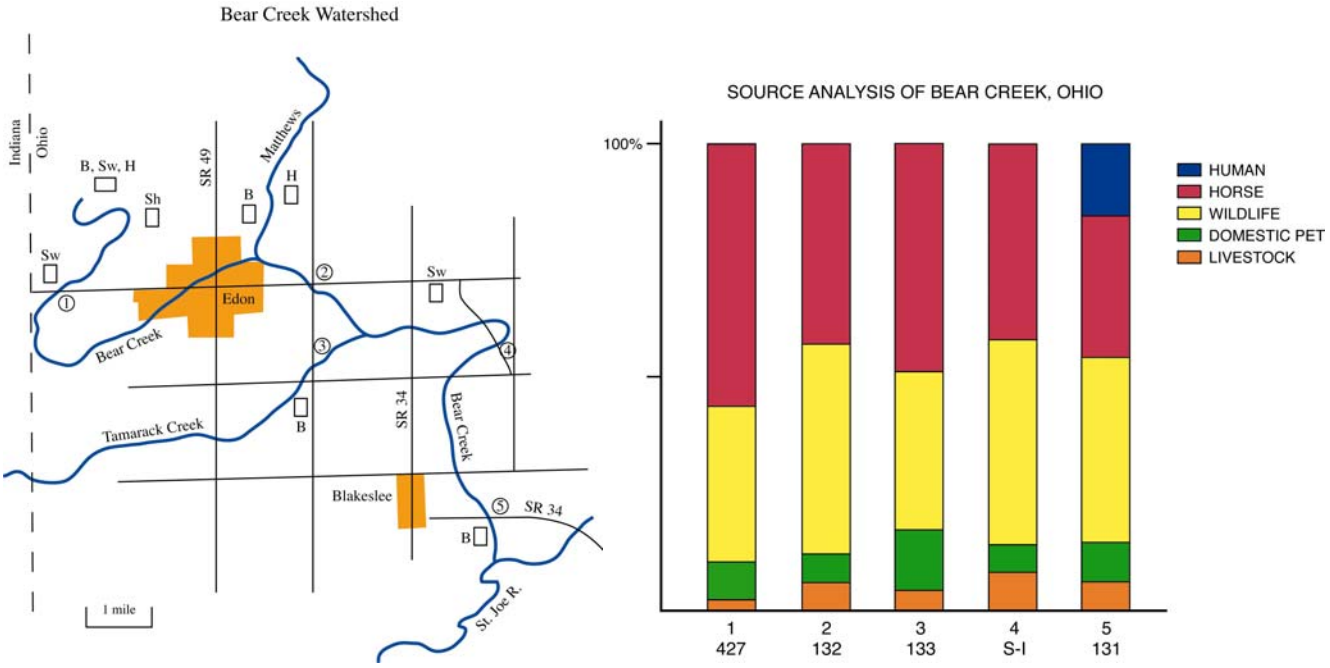
- To perform more extensive sampling in the Cedar Creek sub-watershed in order gather data for the Cedar Creek watershed management plan which was being developed at that time
- To perform more extensive sampling in one additional sub-watershed in order to determine whether sampling closer to sources would change the source distribution.
- To perform multiple samples on a subset (five) of the SJRWI's monitoring sites to determine the effect of time during the sampling season as a factor in the source distribution.
- To determine whether any of the enterococcal strains identified as horse were in fact human strains.

All of the SJRWI's monitoring sites within the Cedar Creek sub-watershed were sampled once during the 2004 sampling season. These included the main stem of Cedar Creek (site 100), Willow Creek (site 101), Black Creek (site 102), Diehl–Peckhart Ditch (site104), Matson Ditch (site 106), Garrett City Ditch (site 117), Diehl Ditch (site136), Walter Smith Ditch (site141), David Link Ditch (site142), and Dibbling Ditch (site143). In addition, sites on Bear Creek (at CR 68) and on Little Cedar Creek (at CR 64, at CR 200S and at SR8) that were not part of the SJRWI's monitoring program were selected for bacterial source tracking. The results of this sampling are reported in Appendix C of this document.

Five sites were selected for repeated sampling: Big Run, OH (127), Garrett Ditch (117), Dibbling Ditch (143), Nettle Creek (144) and Diehl Ditch (136). With the exception of 117, which was not added as a monitoring site until the 2003 season, these sites were sampled over three years. It should be noted that during the 2003 season, the Nettle Creek sampling (2002 site 129) was done a short distance upstream due to construction on the bridge. It is therefore designated as site 144 during the 2003 season. The Diehl Ditch site was dropped from the SJRWI's monitoring sites for the 2004 sampling season, but a 2004 sample was taken for antibiotic resistance analysis. Illustrations of these sampling results can be found in Appendix C, Plate C-1.

While variation occurred at all sites over time, certain trends emerged. Livestock was rarely a significant (greater than 15%) source at the sites; nor was human a significant source. Wildlife was frequently a significant source, often representing the source of the majority of enterococci in the water sample. Horse was often significant as a source, and although it was rarely the source of the majority of strains, it frequently was the source of a substantial (15 - 45%) minority of the strains. Domestic pets were often the source of a substantial minority of the strains.

The Bear Creek (Ohio) sub-watershed was selected for more detailed sampling. Sites were selected based on proximity to known livestock operations and included a site on Rt. 427 west of Edon, Ohio (SJRWI monitoring sites 131, 132 and 133 and BST site 5-I). None of these sites showed a significant contribution by the livestock operation upstream of the site. Horse and wildlife (waterfowl) were consistently the major sources of pollution in the sub-watershed.



The frequency of the horse signature in sampling results prompted concern that overlap with human signature may be hiding possible human source contamination of the water. Within the database, 15.3% of the strains from the human source were found to possess an antibiotic resistance signature identified as horse. In order to determine whether human-horse interference was a problem with the analysis in the watershed, i.e., whether some of the strains identified as horse were in fact human, all of the strains identified as horse from the Cedar Creek samples were subjected to additional testing to determine whether or not they belonged to the species *Enterococcus faecalis*. *Enterococcus faecalis* is only found in humans. This testing consisted of performing a battery of 10 physiological tests on the strains and using an identification key to determine if they were members of the species *E. faecalis.* All of the horse strains in the test were identified through this process as species other than *E. faecalis*, indicating that they were not of human origin, but of animal origin.

To further cross-check results of the antibiotic resistance method of source analysis, twelve 2004 water samples from the Cedar Creek and St. Joseph River watersheds were split at the time of collection and half of each sample was sent to an outside testing lab for source analysis by ribotyping. In this analysis, done by Source Molecular Corporation at their laboratory in Miami, Florida, water samples were cultivated for *E. coli*, and five colonies were randomly selected. The DNA fingerprints of these samples were extracted and were compared to a database to determine whether they were of human or animal origin. Of the twelve samples submitted, four samples contained one isolate each (out of five) that were

identified as human. These were from sites 101, 117, 136, and 142. All others isolates were identified as animal sources. See Appendix E for results of these tests.

While this investigation removes the possibility of human-horse interference, it still does not settle the question of the large contribution of horses to fecal contamination within many areas of the watershed. This appears to be greater than the number of identified horse farms within the watershed; however, there may be many farms in which small numbers of horses are maintained but which have not been identified as horse operations county farm bureau employees. A more detailed census might reveal that horses may be more widespread than has been assumed. Another possibility is that wildlife such as raccoons, opossums, deer, etc. may be giving antibiotic resistance patterns similar to that of horses. Wildlife samples have been difficult to find and this source is not as representative in our database as it perhaps should be based on the rural nature of much of the watershed. Additional investigation is needed in order to determine whether either or both of these possibilities are contributing to produce the observed significant contribution of horses to the fecal load within the watershed.

Conclusions

Some conclusions can be drawn from this research on the use of antibiotic resistance analysis (ARA) to track sources of bacterial contamination in the watershed. Although this research needs to be continued and expanded, ARA has been shown to be valuable in pinpointing the sources of bacterial pollution in watershed streams, especially when combined with land use data.

- The knowledge of land use is an essential component of bacterial source tracking, both in making the decision on which sources to include in the database and also in interpreting data from water samples.

- This study showed that livestock (beef, dairy and swine) contribute little to the overall fecal pollution in the St. Joseph River watershed in terms of percentage of contamination.

- The study also showed that the human contribution of fecal contamination is localized to particular sub-watersheds, and is generally low (not more than 10-15% of the fecal load).

- The study showed that wildfowl make a significant (greater than 50%) contribution to fecal contamination throughout the watershed. This contribution is more pronounced during some parts of the season, but is consistently a major source across the sub-watersheds tested.

- While humans as a source were ruled out as a possible interference with the horse source in the water samples, horse still presents a problem in interpretation.

Given the ability to detect sources along the length of a tributary, it remains very important to thoroughly examine the watershed and its land uses, and then use a combination of BST analysis and land use information to pinpoint pollution sources and work with landowners to find methods to reduce or

eliminate the pollution. Obviously the methods will vary depending upon the source, i.e. reducing the impact of nuisance goose populations is a much different project than eliminating or replacing non-functioning septic systems or fencing livestock from the streams.

Land use data and knowledge of the watershed tells us that horses are not present in significant numbers in most of the sub-watersheds. Horses may be more of a problem within specific sub-watersheds, and/or there may be another source, such as a wildlife source, which is giving an antibiotic resistance pattern similar to that of horses. Expanding the database with additional wildlife samples may help to resolve this difficulty in interpretation.

While this study showed human sources to be small and localized, we do not wish to downplay the importance to human health of eliminating these sources of bacterial pollution from the waters of our streams and ditches. This study did not did not focus on quantifying risk based upon the sources of bacterial contamination. We do not wish to underestimate assessment of the risk of pathogens from animal sources. However, the risk to human health from human pathogens, even in low concentrations, is arguably higher than the risk to humans from pathogens from animal sources. Therefore, elimination of sources of human pathogens in the watershed should be a main focus in watershed planning and restoration.

Recommendations

This project has demonstrated the usefulness of antibiotic resistance analysis in identifying sources of fecal contamination within the St. Joseph River watershed. Some questions still remain, namely the role of horses and wildlife in contributing to the fecal load within the watershed. Additional work on the database as well as more detailed land use analysis could reduce the uncertainty regarding the contribution of these two sources.

Other watersheds in Indiana may find this approach useful. However, databases developed in one watershed cannot be used indiscriminately in other regions. Providing that the agricultural practices and major sources are similar in other watersheds, the database which we developed could be a useful starting point in implementing bacterial source tracking in other watersheds in Indiana. Care must be taken to insure that the database is used appropriately. As a suggestion, interested groups could perform antibiotic resistance analysis on composite samples from each source within the watershed. If these sources are correctly classified by the database, it would be reasonable to add the sources into the database and use it in the new watershed. If not, it would be necessary to develop a database specific for the watershed. It should be pointed out that because of the low cost and time demands of antibiotic resistance analysis, this prospect would not be prohibitively expensive.

As concerns transfer of the database, it currently exists as a file within a specific statistical package (JMPIN). If it is desired to make the database available to interested parties, it would probably be more suitable in a generic spreadsheet format such as EXCEL.

Appendix A: 2002 Sampling Data

Site	Date	Bridge to Water (inches)	% livestock	% pets	% geese	% horse	% human
100a	7/23/2002	336	10.6	12.8	8.5	46.8	21.3
104a	7/23/2002	182	8.5	10.6	0	78.7	2.1
106a	8/6/2002	160	2.1	8.5	27.9	59.6	2.1
123a	7/16/2002	229	11.9	21.4	33.3	31	2.4
124a	7/16/2002	206	16.7	14.4	10.4	50	8.3
125a	7/30/2002	146	6.4	8.5	78.7	4.3	2.1
126a	8/6/2002	193	2.3	14	34.9	39.5	9.3
127a	7/16/2002	198	25	32.5	2.5	40	0
128a	7/16/2002	164	2.1	14.9	8.5	66	8.5
129a	7/30/2002	166	4.2	35.4	56.2	2.1	2.1
130a	7/30/2002	170	4.4	10.9	84.8	0	0
131a	7/16/2002	191	25.5	14.9	29.8	29.8	0
132a	7/30/2002	157	8.7	16.7	41.7	16.7	12.5
134a	8/6/2002	145	13	10.9	71.7	2.2	2.2
135a	8/6/2002	148	2.1	4.3	89.4	4.3	0
136a	7/23/2002	193	4.3	30	27.7	25.5	12.8
137a	7/23/2002	185	0	8.5	21.3	70.2	0
141a	8/6/2002	144	6.3	33.3	35.4	25	0
St Joe a	8/6/2002		6.5	13	39.1	37	4.4
100b	8/20/2002	337	2.1	4.2	91.7	0	0
104b	8/20/2002	180	2.2	6.5	89.1	2.2	0
106b	9/4/2002	160	8.5	14.9	74.5	2.1	0
123b	8/13/2002	230	8.5	6.4	80.9	2.1	2.1
124b	8/13/2002	208	0	4.3	87.2	8.5	0
125b	8/28/2002	149	2.1	2.1	95.8	0	0
126b	9/4/2002	197	9.1	27.3	63.6	0	0
127b	8/13/2002	197	4.2	4.2	79.2	12.5	0
128b	8/13/2002	164	6.5	2.2	82.6	2.2	0
129b	8/28/2002	166	10.6	6.4	57.5	10.6	14.9
130b	8/28/2002	171	0	39.6	25	35.4	0
131b	8/13/2002	192	8.5	4.3	36.2	40.4	10.6
132b	8/28/2002	158	2.1	18.8	39.6	39.6	0
134b	9/4/2002	134	8.3	2.1	89.6	0	0
135b	9/4/2002	148	6.3	12.5	81.3	0	0
136b	8/20/2002	184	6.3	12.8	78.7	0	2.1
137b	8/20/2002	198	0	0	100	0	0
141b	9/4/2002	144	2.1	17	72.3	8.5	0
St Joe b	9/4/2002		8.5	21.7	67.4	2.2	0

Note: Livestock includes beef and dairy cattle, and swin

Appendix B: 2003 Sampling Data

Site	Date	Bridge to Water (inches)	% livestock	% pets	% geese	% horse	% human
100	6/3/2003	340	8.3	4.2	75	0	12.5
104	6/3/2003	176	14.6	4.2	64.6	0	16.7
106	6/10/2003	158	2.1	0	97.9	0	0
117	6/17/2003	63	8.3	8.3	39.6	2.1	41.7
123	6/10/2003	231	4.2	4.3	76.6	0	14.9
124	6/17/2003	197	2.1	10.4	52.1	0	35.4
125	6/24/2003	148	8.3	8.3	79.2	4.2	0
126	6/24/2003	194	18.8	6.3	68.7	6.2	0
127	6/17/2003	196	8.5	6.4	70.2	0	14.9
128	6/10/2003	169	4.2	0	91.7	0	4.2
130	6/17/2003	171	2.1	6.3	33.3	2.1	56.3
131	6/24/2003	193	6.3	4.2	72.9	0	16.7
132	6/24/2003	157	9.5	9.5	69.1	0	11.9
133	6/24/2003	93	2.1	10.6	51.1	0	36.2
134	6/24/2003	134	16.7	10.4	60.4	4.2	8.3
135	6/24/2003	148	8.4	2.1	85.4	0	4.2
136	6/3/2003	183	14.3	2.4	54.8	0	28.6
137	6/3/2003	198	17	2.1	40.4	2.1	38.3
141	6/10/2003	142	2.1	4.2	85.4	0	8.3
142	6/10/2003	132	25	0	72.9	0	2.1
143	6/10/2003	232	6.3	6.3	85.4	2.1	0
144	6/24/2003	139	4.2	4.2	91.7	0	0

Note: Livestock includes beef and dairy cattle, and swine

Appendix C: 2004 Sampling Data

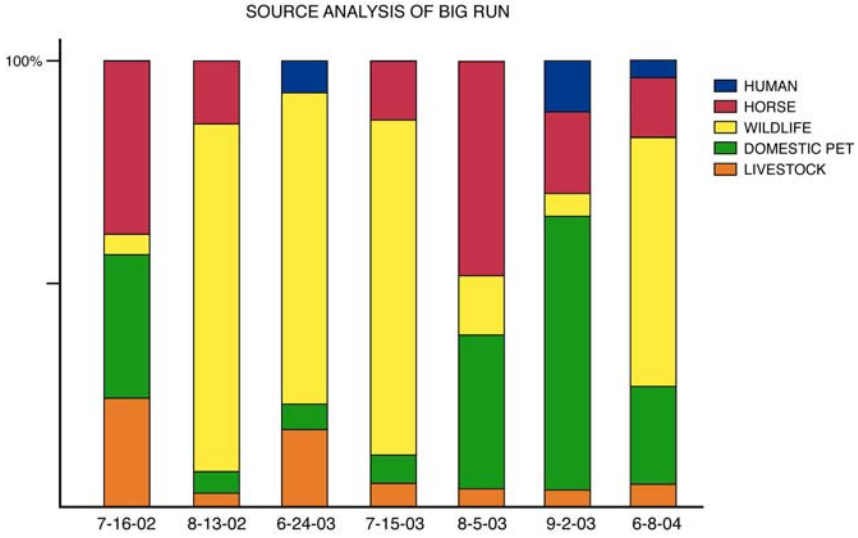
Site	Date	Bridge to Water (inches)	% livestock	% pets	% geese	% horse	% human
100	6/1/2004	285	8.3	16.7	58.3	14.6	2.1
101	6/1/2004	138	14.6	8.3	68.8	2.1	6.2
102	6/1/2004	153	6.2	2.1	77.1	2.1	12.5
104	6/1/2004	165	4.2	6.2	75	0	14.6
106	6/1/2004	133	4.2	6.3	72.9	0	16.7
117	6/8/2004	68	10.6	10.6	40.4	0	38.3
136	6/8/2004	195	10.4	10.4	52.1	0	27.1
141	6/1/2004	152	8.3	12.5	41.7	2.1	35.4
142	6/1/2004	120	2.1	22.9	47.9	2.1	25
143	6/8/2004	220	2.4	24.4	46.3	0	26.8
Bear Creek	6/8/2004		2.1	8.3	62.5	4.2	22.9
LC-CR 64	6/22/2004		0	9.7	48.4	0	41.9
LC-SR 8	6/22/2004		16.7	4.2	35.4	8.3	35.4
LC-CR 200	6/22/2004		28.9	4.4	42	8.9	15.6

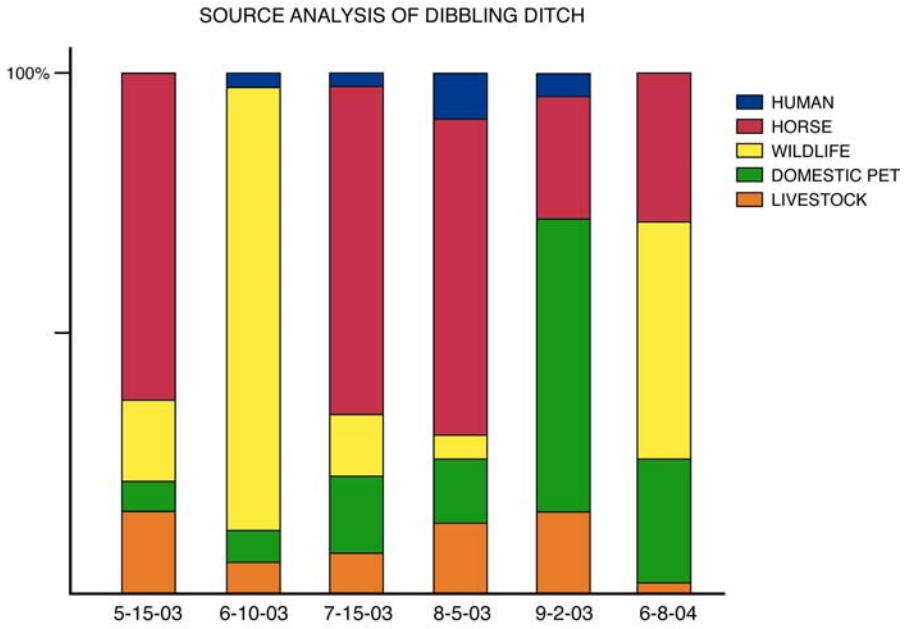
Note: Livestock includes beef and dairy cattle, and swine

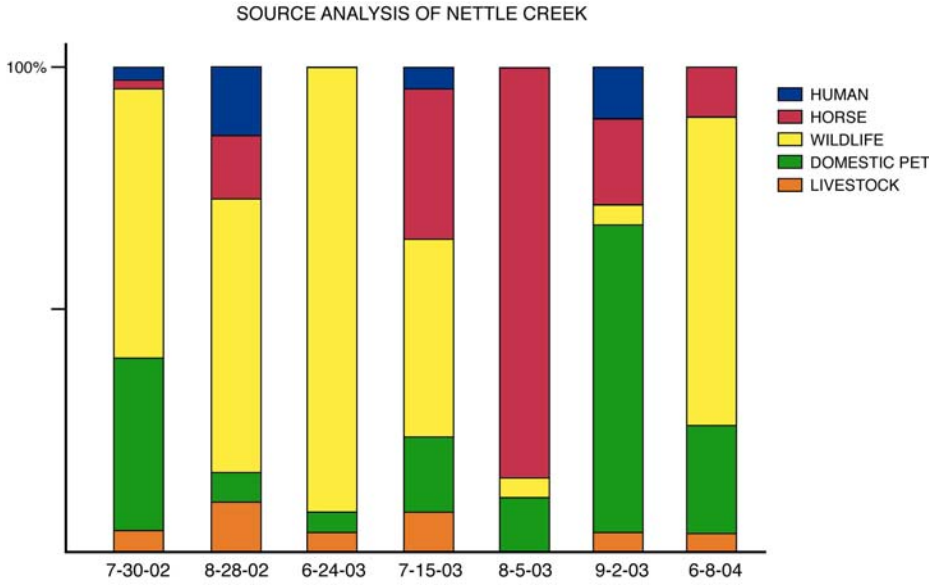
Appendix D: St. Joseph River Watershed Initiative Water Quality Sampling Sites

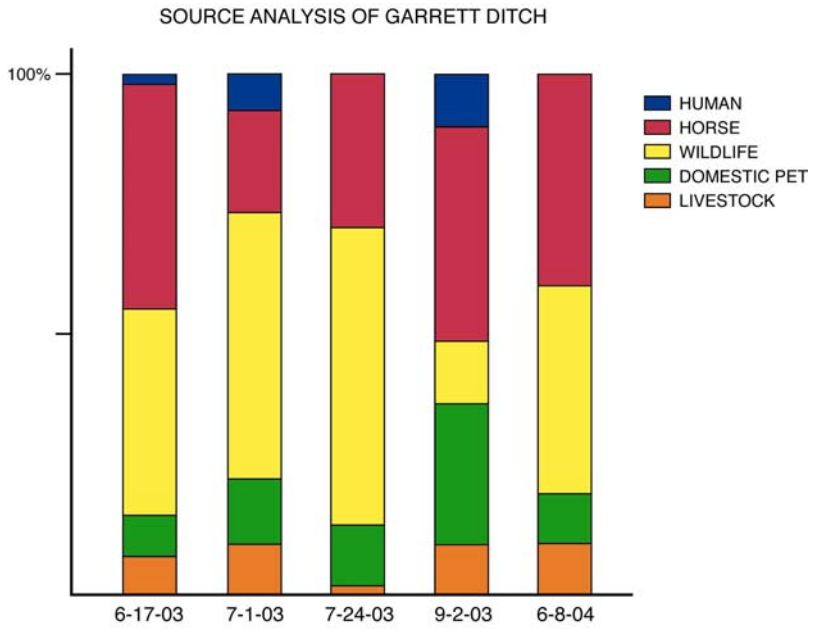
Site #	Location	Name	Site #	Location	Name
100	Tonkel Road	Cedar Creek	123	DeKalb CR 75A	Shank Ditch
101	Coldwater Road	Willow Creek	124	Ohio SR 49	Fish Creek
102	DeKalb CR 7A	Black Creek	125	US 20	St Joe - West
103	DeKalb CR 64	Little Cedar	126	Ohio SR 15	St Joe - East
104	Old SR 427	Diehl/Peckhart	127	DeKalb CR 79	Big Run
105	First Street	Cedar Creek	128	Indiana ST 1	Bear Creek - IN
106	DeKalb CR 39	Matson Ditch	129	Ohio 576	Nettle Creek
107	DeKalb CR 27	Cedar Creek	130	Williams CR J	Eagle Creek
108	Hand Road	Willow Creek	131	Ohio SR 34	Bear Creek - OH
109	Woods Road	Willow Creek	132	Williams CR 4	Matthews Ditch
110	Noble CR 500	South Black Creek	133	Williams CR 4	Tamarack Creek
111	DeKalb CR 68	Little Cedar	134	Sampson Road	East Fork - West
112	DeKalb CR 52	Little Cedar	135	Sampson Road	West Fork - West
113	Indiana SR 8	Diehl Ditch	136	DeKalb CR 19	Diehl Ditch
114	DeKalb CR 40	Peckhart Ditch	137	Indiana SR 8	Peckhart Ditch
115	DeKalb CR 22	Dibbling Ditch	138	Indiana SR 205	Black Creek
116	DeKalb CR 28	Cedar Creek	139	DeKalb CR 40	Diehl Ditch
117	DeKalb CR 15	Garrett City Ditch	140	DeKalb CR 36A	Diehl Ditch
118	Noble Basline Road	Avilla Drain	141	DeKalb CR 39	Walter Smith Ditch
119	DeKalb CR 60	Cedar Creek	142	DeKalb CR 37	David Link Ditch
121	Van Zile Road	St. Joseph River	143	DeKalb CR 18	Dibbling Ditch
122	Halter Road	St. Joseph River	144	Temporary	Nettle Creek

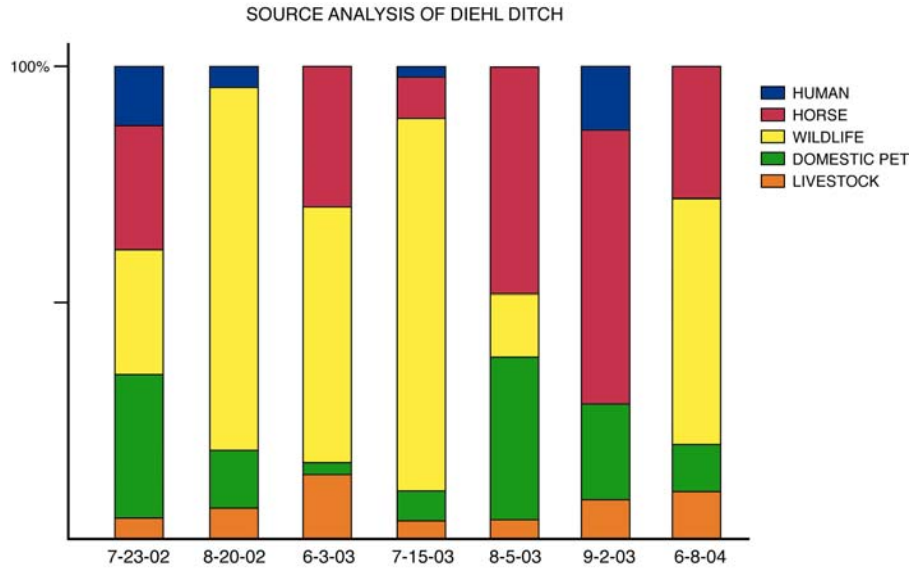
Appendix E: Source Analysis of Five Ditches








SOURCE ANALYSIS OF GARRETT DITCH
100%
HUMAN
HORSE
WILDLIFE
DOMESTIC PET
LIVESTOCK
6-17-03
7-1-03
7-24-03
9-2-03
6-8-04


SOURCE ANALYSIS OF DIEHL DITCH
100%
HUMAN
HORSE
WILDLIFE
DOMESTIC PET
LIVESTOCK
7-23-02
8-20-02
6-3-03
7-15-03
8-5-03
9-2-03
6-8-04

F

Appendix F: Results of 2004 Ribotyping of Samples

Discriminant Analysis of Ribotype Profiles of *E. coli* by Source Molecular Corporation, 4989 SW 74th Court, Miami, FL 33155, USA.

Sample #	Site	Date	Fecal Coliform MPN/100 ml*	*E. coli* Isolate #	Probable Source
P 100 [1]	Cedar Creek Site 100, S R 427, Tonkel Road, Allen County	June 1, 2004	> 2,400	1 2 3 4 5	Animal Animal Animal Animal Animal
P 101 [2]	Willow Creek Site 100, S R 327 Coldwater Road, Allen County	June 1, 2004	>2,400	1 2 3 4 5	Human Animal Animal Animal Animal
P 102 [1]	Black Creek, DeKalb CR 7A	June 1, 2004	>2,400	1 2 3 4 5	Animal Animal Animal Animal Animal
P 104 [1]	Diehl-Peckhart Ditch, Old S R 427, DeKalb County	June 1, 2004	>2,400	1 2 3 4 5	Animal Animal Animal Animal Animal
P 106 [1]	Matson Ditch, DeKalb CR 39	June 1, 2004	>2,400	1 2 3 4 5	Animal Animal Animal Animal Animal
P 141 [1]	Walter Smith Ditch, DeKalb CR 39	June 1, 2004	>2,400	1 2 3 4 5	Animal Animal Animal Animal Animal
P 142 [2]	David Link Ditch, Dekalb CR 37	June 1, 2004	>2,400	1 2 3	Animal Animal Animal

				4 5	Animal Human
117-04 [2]	Garrett City Ditch, DeKalb CR 15	June 8, 2004	>2,400	1 2 3 4 5	Human Animal Animal Animal Animal
127-04 [1]	Big Run, DeKalb CR 79	June 8, 2004	> 2,400	1 2 3 4 5	Animal Animal Animal Animal Animal
129-04 [1]	Nettle Creek, Ohio SR 576	June 8, 2004	= 1,100	1 2 3 4 5	Animal Animal Animal Animal Animal
136-04 [2]	Diehl Ditch, DeKalb CR 19	June 8, 2004	= 240	1 2 3 4 5	Animal Human Animal Animal Animal
143-04 [1]	Dibbling Ditch, DeKalb CR 18	June 8, 2004	=1,100	1 2 3 4 5	Animal Animal Animal Animal Animal

* mpn – most probable number of fecal coliforms in 100 mL of sample after 20 hrs. of cultivation at 44.5° C.

[1]Laboratory Comments: The DNA fingerprints of the five colonies of *E. coli* cultured from the water sample statistically matched animal sources recorded in a database of known source DNA fingerprints. The results do not represent that animal *E. coli* is the only *E. coli* in the system under investigation. Further analysis of multiple samples from multiple locations, in repetition would add further confidence. Pinpointing the source of animal *E. coli* is possible by collecting fecal matter from the predicted sources along with further contaminated water samples, and then looking for a direct DNA match.

[2]Laboratory Comments: The DNA fingerprints of the five colonies of *E. coli* cultured from the water sample statistically matched both human and animal sources when compared to a database of known source DNA fingerprints. Pinpointing the source of animal *E. coli* is possible by collecting fecal matter from the predicted sources along with further contaminated water samples, and then looking for a direct DNA match.

APPENDIX G

Bacterial Source Analysis of Water Samples

Filtering of Water Samples

Within 6 hours of sample collection, samples are filtered using a sterile filter apparatus and presterilized Gelman GN-6 filters (0.22 micron poor size). Amount of water to be filtered depends on the investigator's estimate of the numbers of bacteria in the sample. For pristine water, two 25 mL and one 50 mL samples may be used, while for more polluted water, 2 5 mL and one 10 mL samples may be used. After filtration, the filter is placed on the surface of mENT agar in a Petri dish using a sterile forceps. The plates are incubated for 48 hours at 37^{o}C.

Isolation of Fecal Enterococci

After 48 hours incubation, red colonies are picked off of the filters using sterile toothpicks and inoculated into wells of a microwell plate containing Enterococcosel broth. 48 colonies are transferred per sample. Plates are incubated for 24 hours at 37^{o}C.

Antibiotic Resistance Testing

Media containing antibiotics is made up at least one day in advance by adding various amounts of antibiotic stock solutions to 100 mL of sterile TSA to give the target concentration.

Antibiotics and their concentrations which are routinely used are:

Control	
Tetracycline	10, 10, 30, 50, 100 ppm
Chlortetracycline	60, 80, 100 ppm
Oxytetracycline	20, 40, 60, 80, 100 ppm
Neomycin	40, 60, 80 ppm
Cephalothin	10, 15, 30, 50 ppm
Erythromycin	10, 15, 30, 50 ppm
Streptomycin	40, 60, 80, 100 ppm
Vancomycin	2.5 ppm

Amoxicillin 0.156 ppm

Microwells are examined for the presence of a black color due to hydrolysis of bile esculin, which is diagnostic for enterococci. Any wells not turning black are noted and are not used in the data analysis. Plates are inculated by replica plating with a flame sterilized metal replicator containing 48 prongs. The replica plater is dipped into the microwells and then placed on top of the agar surface to transfer the bacteria. After drying, the plates are inverted and incubated for 48 hours at 37^{o}C.

Data Recording

Growth of each isolate on each antibiotic is recorded as positive or negative on record sheets.

Data Analysis

Data are analyzed by discriminate analysis using the JMP-IN program.